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DOI: https://doi.org/10.1128/AAC.05919-11

AAC Accepts, published online ahead of print on 6 February 2012
Antimicrob. Agents Chemother. doi:10.1128/AAC.05919-11

Ajoene, a Sulfur Rich Molecule from Garlic, Inhibits Genes Controlled by Quorum Sensing

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Running title: QSI activity of ajoene

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Abstract

In relation to emerging multiresistant bacteria, development of antimicrobials and new treatment strategies of infections should be expected to become a high priority research area. Quorum Sensing (QS), a communication system used by pathogenic bacteria like *Pseudomonas aeruginosa* to synchronise the expression of specific genes involved in pathogenicity, is a possible drug target. Previous *in vitro* and *in vivo* studies revealed a significant inhibition of *P. aeruginosa* QS by crude garlic extract. By bioassay-guided fractionation of garlic extracts we determined the primary QS inhibitor present in garlic as ajoene, a sulfur-containing compound with potential as an antipathogenic drug. By comprehensive *in vitro* and *in vivo* studies of the effect of synthetic ajoene towards *P. aeruginosa* was elucidated. DNA microarray studies of ajoene treated *P. aeruginosa* cultures revealed a concentration dependent attenuation of a few, but central QS controlled virulence factors including rhamnolipid. Furthermore, ajoene treatment of *in vitro* biofilms demonstrated a clear synergistic, antimicrobial effect with tobramycin on biofilm killing and a cease in lytic necrosis of polymorphonuclear leukocytes. Furthermore, in a pulmonary infectious mouse model a significant clearing of infecting *P. aeruginosa* was detected in ajoene-treated mice compared to a non-treated control group.

This study adds to the list of examples demonstrating the potential of QS interfering compounds in the treatment of bacterial infections.

Introduction

Infections that develop into chronic conditions are a fast growing problem in the developed world. The underlying biology is thought to be the ability of bacteria to form biofilms (20) which consist of structured and aggregated (often surface-attached) communities of bacteria (18). Multiple studies have documented that such aggregated communities are more resistant to a variety of antibiotics and the action of the immune system compared to their planktonic counterparts (6, 19, 41, 68). Biofilm infections are often connected to patients with medical devices and implants as well as hospitalized patients. Lately bacterial biofilms have also been associated with non-healing, chronic wounds (9, 24, 38). There is thus an urgent need for development of new treatment strategies using a combination of drugs targeting a multitude of antimicrobial targets.

Several Gram-negative pathogens use *N*-acyl homoserine lactones (AHLs)-mediated communication systems in a process termed quorum sensing (QS) to coordinate specific gene expression, thereby synchronizing expression of particular phenotypic features between the individual cells (28). QS is thought to play an important role during the initial event of infection for the common opportunistic Gram-negative human pathogen *Pseudomonas aeruginosa*, which is associated with nosocomial and wound infections, immunocompromised (48, 70) and the genetic inherited disease cystic fibrosis (CF) (22). By employing the QS system to control expression of its virulence factors (many of which are antigenic determinants), *P. aeruginosa* is able to operate in a stealthy manner

until a certain cell density is reached where the QS systems become activated. Upon activation of the QS systems, a coordinated release of tissue damaging and immune defense degrading virulence factors takes place (28, 36). It was recently documented by us that the QS controlled virulence factor rhamnolipid (also known as heat-stable hemolysin) destroys polymorphnuclear (PMN) leukocytes by lytic necrosis (36). Besides lysing neutrophils and macrophages, rhamnolipid has also been reported to impair chemotaxis of neutrophils (42, 62). As rhamnolipid is associated with bacteria living in biofilm, it is likely to function as a shield towards important cellular components of the host defense (1, 36, 71). Furthermore, QS promotes increased tolerance of *P. aeruginosa* biofilms to antibiotic treatments (6) and provides biofilms with structural rigidity through release of eDNA (21, 56).

Two of the *P. aeruginosa* quorum sensors are based on the LuxRI homologues present in most Gram-negative bacteria with QS systems. The I homologues function as an AHL synthetase producing the required signal molecules and the R homologues function as transcriptional activators which upon binding of the cognate signal molecules activate the transcription of the QS target genes (28). The *P. aeruginosa* QS system consists of *lasRI* and *rhlRI* hierarchically arranged with the *las* encoded system normally at the top level controlling the *rhl* encoded system and a third system intervened in between the two denoted the pseudomonas quinolone signal (PQS). The three interacting QS systems LasRI, RhlRI and PQS use the following signal molecules for activation, *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), *N*-butanoyl homoserine lactone (C4-HSL) and 2-heptyl-3-hydroxy-4-quinolone (PQS), respectively (50, 51, 54).

92 It has been shown that some terrestrial and marine organisms have evolved a production
93 of specific molecules with AHL-antagonistic activity capable of interfering with the
94 bacterial QS system in a possible prevention of colonization (8, 55, 58, 59, 64). Hentzer
95 et al. (33) and Wu et al. (76) demonstrated that the QS system could be used as an
96 effective antimicrobial drug target by altering the tolerance of biofilms to antibiotic
97 treatment and promote a faster clearance of a *P. aeruginosa* lung infection in mice by
98 using the chemically modified QS inhibitor (QSI) furanone C-30. Crude extracts of garlic
99 (*Allium sativum L*) have been shown to inhibit the expression of a large number of QS
100 controlled genes (58) and Bjarnsholt et al. (8), demonstrated the ability of garlic extracts,
101 similar to C-30 treatments, to promote a rapid clearing of a pulmonary *P. aeruginosa*
102 infection in mice. Garlic is widely accepted as a herb that through a dietary intake can
103 improve human health (61). Epidemiological studies have shown that a daily intake of
104 garlic lowers the risk of certain cancers (25, 63) and several studies have documented an
105 antithrombotic and lipid lowering cardiovascular effect of some of the constituents in
106 garlic (27).
107 By means of a bioassay directed purification procedure, we identified the sulfur
108 containing compound ajoene (4,5,9-trithiadodeca-1,6,11-triene 9-oxide) as a QSI present
109 in garlic extract. When garlic is crushed, ajoene and several other organosulfides are
110 produced as degradation products of allicin (diallyl thiosulfinate) (11). Ajoene has been
111 reported to display conventional antimicrobial activities to a number of Gram-positive
112 bacteria and the Gram-negative bacteria *Escherichia coli, Klebsiella pneumoniae* and
113 *Xanthomonas maltophilia* but not *P. aeruginosa* (45). To further exploit the QSI activity
114 *in vitro* and *in vivo*, we employed chemically synthesized ajoene (M. Givskov, 8

December 2010, European Patent Application, No. 10194154.0 and US provisional application, No. 61/420,922). The *in vitro* experiments showed significant inhibition of a subclass of QS-regulated *P. aeruginosa* genes and a significant synergistic action with tobramycin with respect to the reduction of viability of biofilm cells. Furthermore, a pulmonary infectious mouse model was employed to demonstrate the antimicrobial effect of ajoene on *P. aeruginosa* infections.

Materials and methods

Bacterial strains

Sequenced *P. aeruginosa* PAO1 wild-type obtained from the Pseudomonas Genetic Stock Center (www.pseudomonas.med.ecu.edu, PAO0001). For detection of QSI activity following reporter strains were used; QSI selector 1 strain (QSIS1, *E. coli*), *lasB-gfp*, *rhlA-gfp* (*P. aeruginosa*) and *luxI-gfp* (*E. coli*) monitor strains described in (58), (32), (77) and (4) respectively. Production of AHL's was detected by using the following reporter strains; *lasB-gfp* (*E. coli*) (32) and *ahyI-gfp* (*E. coli*) (30). Animal experiments were performed with the wild-type *P. aeruginosa* strain (PAO1) obtained from Professor Barbara Iglewski (University of Rochester Medical Center, NY, USA). The strain is QS-proficient, except for reduced production of C4-HSL previously noted for this *P. aeruginosa* variant (39). The clinical isolate CF438 was obtained from a child with CF and kindly provided by Helle K. Johansen and Oana Ciofu. The mucoid and non-mucoid isogenic strains are described in (44, 72).

Growth media and conditions for *in vitro* and *in vivo* experiments

BT minimal medium (B medium (17) plus 2.5 mg thiamine l^{-1} and 10% A10 (17)) supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol) Casamino acids were used for growing the monitor strains (overnight cultures) for the QSI indicator screens. All strains were incubated at 30ºC with shaking (180 rpm) and supplemented with antibiotics where appropriate. For animal experiments bacteria from freezer stocks were plated onto blue agar plates (State Serum Institute, Denmark) and incubated at 37ºC overnight. Blue agar plates are selective for Gram-negative bacilli (34). One colony was used to inoculate overnight cultures grown in Luria–Bertani (LB) medium at 37ºC with shaking.

Extraction and purification of QS containing fractions from garlic

Fractions containing QS activity were identified by a QSIS1 bioassay guided fractionation according to (58). Garlic cloves were skinned, homogenized in toluene and stirred overnight with an equal volume of water. The garlic pulp was filtered and the solvents separated, dried under vacuum and tested for QSI activity. Activity was only observed from the toluene extract. The extract was fractionated on a C_{18} column (125g, 200 x 50 mm) on a Biotage® Isolera™ flash purification system (Isolera) with a flow rate of 30 mL/min with sample added as dry load. Samples were collected (without detection) as 100 mL fractions, first sample at 10% MeOH in H_2O, the following 10 samples were eluted with a 10-100% MeOH gradient (1000 mL) and all subsequent samples 100% MeOH (typically 500 mL). Activity was detected in the 50-60% MeOH fractions, which were combined and further purified by semi preparative HPLC on a Chromolith RP-18e column (100 x 4.6 mm) with a flow rate of 2 ml per min, eluting with 30% MeCN and increasing to 37% MeCN over 20 min. A single fraction eluting at 9.5 min was

161 determined to have QSI activity. Positive electrospray (ESI^+) high-resolution mass
162 spectrometry (HRMS) gave a mass of 235.0282 Da, corresponding to a formula of
163 $C_9H_{15}OS_3$. Comparison of 1H-NMR data identified this fraction as a 60:40 mixture of *E*
164 and *Z* ajoene (11).
165

166 ## Chemically synthesized ajoene

167 Ajoene was synthesized from commercially available distilled allyl disulfide as a 1:4
168 mixture of E:Z isomers according to (M. Givskov, 8 December 2010, European Patent
169 Application, No. 10194154.0 and US provisional application, No. 61/420,922). Synthetic
170 ajoene was purified by silica gel chromatography and characterized by 1H-NMR, 13C-
171 NMR and HRMS. The purity was greater than 98%. Synthetic ajoene was used in all
172 experiments conducted in this paper.
173

174 ## Determination of inhibitor strength

175 The following bioassays were used to determine inhibitor activity of ajoene. ABT
176 medium (150 µl) supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol) Casamino
177 acids were added to all wells in a 96 wells microtiter dish (Black Isoplate®, Perkin
178 Elmer). To the first column ajoene is added to a final concentration of 200 µg/ml and a
179 twofold serial dilution is made. No ajoene was added to the last column, which was used
180 as a reference. At last, 150 µl overnight cultures of the QSI monitors (*lasB-gfp* (32), *rhlA-*
181 *gfp* (77) or *luxI-gfp* (4)) were added to all the wells to a final OD_{450nm} on 0.1.
182 Additionally, the signal molecule OHHL was added to the *luxI-gfp* reporter screen in a
183 final concentration of 100 nM. The growth of the bacteria cells (OD_{450}) and the GFP

184 expression (excitation wave length, 485 nm and emission wave length, 535 nm) was
185 measured on a multilabel plate reader Wallac 1420 VICTOR^2 (Perkin Elmer) every 15
186 min over 14 h. The temperature was held constant at 34ºC.
187

188 ## Production of QS signal molecules

189 Production of C4-HSL and 3-oxo-C12-HSL was detected in the supernatant from an
190 overnight culture of clinical isolate (CF438) by using the AHL-specific reporter strains
191 and the method described by Hentzer et al., (32).
192

193 ## RNA preparation for DNA microarray analysis

194 Exponential growing (OD_{600nm} of 0.5) *P. aeruginosa* PAO1 at 37°C, 180 rpm in AB-
195 media supplemented with 0.5% Casamino acid were diluted to an OD_{600nm} of 0.05. When
196 reaching OD_{600nm} of 0.5 the culture were divided into 5 cultures of 50 ml and the
197 following four concentrations of ajoene were added; 10 µg/ml, 20 µg/ml, 40 µg/ml and
198 80 µg/ml and to one culture no ajoene was added. At OD_{600nm} of 2.0 samples were
199 retrieved and two volumes of RNAlater (Ambion) were added. Isolation of RNA was
200 performed using the RNeasy Mini Purification Kit (Qiagen) with on-column DNase
201 treatment. The following synthesis of cDNA and hybridization were performed by the
202 microarray core-unit at Rigshospitalet, Denmark. The gene expressions were analyzed by
203 the use of the software ArrayStar v3.0 (DNAstar). DNA microarray analysis of global
204 gene expression was performed according to guidelines provided by Affymetrix and
205 repeated three times with RNA from three individual growth experiments.
206

Real Time PCR

The purified RNA used for DNA microarray was also used for RT-PCR. cDNA was made from 1 µg of RNA using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). For quantitative real-time PCR, amplification was performed with Power SYBR Green Master Mix in a Step One Plus Thermal Cycler (Applied Biosystems). The primers were designed using Primer Express3.0 (Applied Biosystems). Forty cycles were run with denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 60°C for 45 seconds. The gene *rpoD* was used as control for standardization. Primer sequences: *rhlA* forward; 5'-GGCGATCGGCCATCT-3', *rhlA* reverse; 5'-AGCGAAGCCATGTGCTGAT-3', *lasB* forward; 5'-CGACAACGCGTCGCAGTA-3', *lasB* reverse; 5'-AGGTAGAACGCACGGTTGTACA-3'. *rpoD* forward; 5'-ACAAGATCCGCAAGGTACTGAAG-3', *rpoD* reverse; 5'-CGCCCAGGTGCGAATC-3'.

Measurements of total rhamnolipid production

Samples for measurements of total rhamnolipid concentration were retrieved from the cultures grown for DNA-microarray and RT-PCR at OD_{600nm} of 1.5 and 2.0 and kept at -80°C until further examinations. HPLC with ESI^+-HRMS detection (47) was used to quantify rhamnolipids as their $[M+NH_4]^+$ (peak area) based on external standard quantification of a NMR validated rhamnolipid B standard. A series of diluted standard was analyzed before and after the samples, in order to minimize potential differences in ionization levels of rhamnolipid between the samples. Other rhamnolipids were assumed to give the same ionization efficiency as rhamnolipid B. Total rhamnolipid concentration

was derived from the six major rhamnolipids, with the following masses $[M+NH_4]^+$: 668.4 (rhamnolipid B, C10-C10-rha-rha), 694.4 (C10-C12D-rha-rha), 696.4 (C10-C12-rha-rha), 522.4 (C10-C10-rha), 548.4 (C10-C12D-rha); and 550.4 (C10-C12-rha).

Stability of ajoene

Overnight cultures of the following strains; *P. aeruginosa* PAO1, Δ*lasR*-Δ*rhlR and* Δ*lasI*-Δ*rhlI* were diluted to OD_{600nm} of 0.2 and incubated with 100 µg/ml ajoene at 37°C and 4°C for 18 h. The samples were sterile filtrated (0.22 µm pore size) and to measure QSI activity of ajoene the supernatant were tested in the *P. aeruginosa* QSI screen using the *lasB-gfp* monitor strain (see "Determination of inhibitor strength").

Effect of ajoene on *P. aeruginosa* QS signal molecule content

Production of AHL was quantified by HPLC with tandem mass spectrometry (MS/MS) as described in (60). *P. aeruginosa* was grown as for DNA-array and RT-PCR with the same concentrations of ajoene added. At OD_{600nm} of 1.5, 1.8 and 2.0, samples were retrieved and sterile filtrated (0.22 µm pore size). Acidified ethyl acetate was added to the supernatant in a 1:1 ratio and left at room temperature over night. The top phase was withdrawn and concentrated under nitrogen gas and resuspended in 500 µl EtOH, with 1 µl analysed by HPLC-MS/MS (60). In this case external standard quantification was done. The method detects C4-HSL, open lactone-C4-HSL, 3-oxo-C12-HSL, and open lactone-3-oxo-C12-HSL. Detection limits were in the 10–30 nM range.

Effect of serum albumin on the QSI activity of ajoene

The *P. aeruginosa* QSI screen (*lasB-gfp*) was used to test the effect of serum albumin on ajoene activity. Bovine serum albumin was dissolved in the ABT-media to a concentration of 100 mg/ml. 300 µl were added to the first row of a 96 wells microtiter dish (Black Isoplate®, Perkin Elmer). To the rest of the rows 150 µl media without serum albumin were added. A 2 fold serial dilution of serum albumin was made and ajoene added to the following final concentrations; 12.5 µg/ml and 25 µg/ml. Finally 150 µl of the *lasB-gfp* monitor strain were added to all the wells (for a more detailed description see "Determination of inhibitor strength").

In vitro biofilms

Biofilms were grown at 37ºC in continuous-culture, once-through, three channel, flow cells with individual channel dimensions of 1*4*40mm perfused with sterile AB trace minimal medium containing 0.3 mM glucose as described by Christensen et al. (15) and Pamp and Tolker-Nielsen (49). Overnight cultures were diluted to 0.1 at OD_{600nm} in 0.9% NaCl, and 250 µl was used for inoculation per channel. All microscopic observations and image acquisitions were performed using a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems, Germany). Images were obtained with an x40/dry objective and x100/oil objective. To visualize dead bacterial cells and lysed PMNs, propidium iodide (PI) (P-4170; Sigma) was used, whereas expression of GFP was used as a measure for live bacterial cells. Image scanning was carried out at 488 nm (green) and 543 nm (red) laser line from an Ar/Kr laser. Imaris software package (Bitplane AG) was used to generate pictures of the biofilm. Tobramycin was diluted in 0.9% NaCl. The medium containing ajoene was kept on ice during the experiment.

Preparation of PMNs

Isolation of PMNs was performed according to Bjarnsholt et al. (6) with modifications. Human blood was collected from normal healthy volunteers in BD Vacutainers containing 0.129 M sodium citrate. PMNs were resuspended in RPMI 1640 with $NaHCO_3$ to obtain a concentration of $1.5*10^7$ PMNs/ml.

PMN exposure of biofilms

The exposure experiment was performed as described by Bjarnsholt et al. (6). We evaluated the biofilm and PMN interactions every 30 min for 2 hours. Necrotic PMNs were demonstrated as increased red fluorescence from the supplemented DNA stain PI.

Animals

Female BALB/c mice were purchased from Taconic M&B A/S (Ry, Denmark) at 9-11 weeks of age and were maintained on standard mouse chow and water ad libitum for a minimum period of 1 week before the challenge.

The animal studies were carried out in accordance with the European convention and directive for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and the Danish law on animal experimentation. All experiments were authorized and approved by the National Animal Ethics Committee, Denmark (The Animal Experiments Inspectorate, dyreforsoegstilsynet.dk), and given the permit number 2008/561-1466. All surgery was performed using hypnorm/midazolan and pentobarbital

was used to euthanize the mice at the termination of the experiments. All efforts were made to minimize suffering.

Pulmonary infection model

The pulmonary infection model in mice was prepared and performed according to (10). The ajoene solution used for the treatment group was prepared as follows: ajoene was dissolved in 96% ethanol to a concentration of 100 mg/ml and diluted 40x in a 20% vehicle solution ((2-hydroxypropyl)-β-cyclodextrin (Sigma, cat. no. C0926) dissolved in 0.9% NaCl) to a concentration of 2.5 mg/ml reducing the concentration of ethanol to 2.4%. Each mouse were treated with 25 mg ajoene kg^{-1} BW subcutaneously (s.c.) once a day as prophylactic treatment for two days, right after infection and subsequently two days post-infection. The placebo group received 96% ethanol diluted in the vehicle corresponding to the amount of ethanol that the ajoene treated group received.

Statistical Analysis

The number of mice in each group was calculated to provide a power of 0.8 or higher for continuous data. For analysing quantitative data the Mann-Whitney U test was used for calculating p-values in the statistical program GraphPad Prism (GraphPad software, Inc., San Diego, USA, version 5.0). P-values ≤ 0.05 were considered significant.

Apoptosis assay

Apoptosis tests in the lung epithelial cell line A549 (purchased from the German Collection of Microorganisms and Cell Lines DMSZ, Braunschweig, Germany) was

performed by flow cytometry using a fluorescein isothiocyanate [FITC]-conjugated antibody to cleaved Poly [ADP –ribose] polymerase (PARP) (Cell Signaling Technology, Denver, CO, USA). Samples of 5×10^5 cells were incubated in the presence of various concentrations of ajoene in six-well plates for 4 h. Subsequently, cells were detached with trypsin/EDTA, washed twice and fixed in 2% formaldehyde for 15 min, permeabilized by 0.1% Saponin (Roth GmbH, Karlsruhe, Germany) in PBS for 60 min and incubated with anti-cleaved-PARP according to the manufacturer's protocol for 30 min. For positive control, cells were cultured in the presence of apoptosis inducer tetrandrine (Sigma Aldrich, Steinheim, Germany) for 4 h (12).

Proliferation assay

Interference of ajoene with A549 cell proliferation was measured employing an MTS proliferation assays (Promega, Mannheim, Germany) following the manufacturer's protocol.

Toxicity assay

Cytotoxicity testing was performed using a lactate dehydrogenase release based assay kit purchased from Roche Applied Science (Mannheim). A549 lung epithelial cells were exposed to concentration series of test substances for 24 h before LDH release was determined according to the manufacturer's instructions.

Results

Ajoene is the major bioactive QSI compound in garlic extract

Previously demonstrations of inhibition of QS in *P. aeruginosa* with crude and partially purified garlic extracts (8) encouraged us to further identify and assess the efficacy of the pure QS inhibitor. In our hands, Spanish garlic appeared to contain a higher level of bioactivity with respect to QS inhibition, compared to those obtained from other countries including China and Argentina (data not shown). The compounds present in garlic bulb extracts were stable to time, protease activity and various evaporation techniques at room temperatures, and it was only at high temperatures that degradation was observed. An iterative process of fractionation and assaying was applied to crude garlic extracts to determine the presence of any potential QSI compounds (bioassay-guided fractionation). Several different water extracts were investigated, initially testing several different columns for fractionation and purification. These columns included matrices consisting of C_{18}, Sephadex G10 and LH20, and HILIC (hydrophilic interaction chromatography), however, in the end ajoene was isolated on C_{18} material, as described in the materials and methods section. During examination of the initial water extract, the activity was lost. Investigations showed that the active compound was most likely degraded or adsorbed to sodium sulfate (used as a drying agent). Consequently, the focus was switched to the toluene phase. The toluene extracts continuously showed a high level of QSI activity. The treatment of these extracts was similar to that of the water extracts, however all drying agents were avoided in case of absorption of the active compounds. In this case the primary column matrixes used were DIOL, Silica gel (Si) and C_{18}. Bioassay-guided fractionation based on Quorum Sensing Inhibition Screens (QSI Screens: QSIS1, *lasB-gfp* and *rhlA-gfp*) of these extracts isolated a single primary compound responsible for the *in vitro* activity. This compound was isolated and examined with Mass Spectrometry

(MS) and Nuclear Magnetic Resonance (NMR) (data not shown) and identified as ajoene (Fig. 1). In addition several ajoene derivatives (Fig. 1) were shown to be present. Ajoene is a lipid-soluble allyl sulfide formed from allicin which is converted from alliin by an enzymatic process when garlic is crushed (11). A range of different organosulfur compounds are formed in this process with ajoene being among of the most abundant (37). To further investigate the QSI bioactivity, we used chemically synthesized ajoene prepared by a recently published method (M. Givskov, 8 December 2010, European Patent Application, No. 10194154.0 and US provisional application, No. 61/420,922). Both naturally occurring and chemically synthesized ajoene exist as two isomers, (Z) and (E), in different ratios dependent on the preparation method (Fig. 1).

To determine the QSI activity of ajoene, dose-response curves were created using two QSI reporter systems which contain fusions of the QS controlled *lasB* promoter and *rhlA* promoter to *gfp*(ASV) encoding an unstable GFP variant in a *P. aeruginosa* background (32, 77). We also used a QS reporter system harbored in an *E. coli* background where the *luxR* gene and the promoter region of the *luxI* is fused to *gfp*(ASV) (4). In all three reporter systems, induction of the QS system can be measured as increasing fluorescence. The presence of an antagonist decreases GFP expression and thus fluorescence is proportional to the concentration or effectiveness of the QSI present. Growth of the reporter strains was monitored to make sure that the concentrations of added ajoene were not affecting primary metabolic functions and thereby altering growth rate. IC_{50} values were calculated from the curves expressing the specific fluorescence (Gfp expression/cell density) (Fig. 2), giving the following values: *lasB-gfp*, 15 µM; *rhlA-gfp*, 50 µM and *luxI-gfp*, 100 µM. The calculations were performed by plotting the maximal slopes from the

curves obtained with the different reporter strains as a function of the concentrations of ajoene. The slope represents the synthesis rate ($\Delta RFU/OD_{450}/\Delta time$).

Target gene specificity

DNA microarray analysis was used to identify the target gene specificity of ajoene. As a reference we have used the QS regulon previously identified by Hentzer et al. (33). In the past this dataset has been used to validate target specificity of putative QSI compounds. The study by Hentzer et al. (33) defines QS regulated genes as those genes for which the expression is altered more than 5-fold in a Δ*lasI*-Δ*rhlI* mutant in response to the addition of exogenous C4-HSL and 3-oxo-C12-HSL. Genes with less than 5-fold alteration in expression between treated and non-treated cultures were not included in this study. Exponentially growing *P. aeruginosa* cultures were treated with the following four concentrations of ajoene: 10 µg/ml (42.7 µM), 20 µg/ml (85.4 µM), 40 µg/ml (170.8 µM) and 80 µg/ml (341.6 µM), none of which affect growth (see *supplemental material*, Fig. S1). The samples were retrieved at an OD_{600nm} of 2.0 (optical density) as previous investigations have shown the highest activity among the QS genes at this particular cell density.

Only a small number of genes were significantly ($p<0.05$) more than 5-fold down-regulated by the four different concentrations of ajoene; 0 (10 µg/ml), 0 (20 µg/ml), 2 (40 µg/ml) and 11 (80 µg/ml). According to the QS regulon defined by Hentzer et al., 10 of the 11 genes and according to Rasmussen et al. (59), all the genes down regulated more than 5-fold by ajoene are defined as QS regulated. There is a clear relationship between an increasing concentration of ajoene used for treatment and the degree of regulation of

the target genes. The transcriptomic analysis indicated that the optimum concentration for repression of the target genes is close to 80 µg/ml ajoene. Attempts to repress more than those 11 genes (out of a total of 5570 *P. aeruginosa* genes) were not possible without supplementing cultures with concentrations that would also affect growth. This means that ajoene administered at this optimum concentration exhibits a high degree of target specificity towards a small subgroup of the QS regulon. Five genes were significantly ($p<0.05$) more than 5-fold up regulated in response to treatment with 80 µg/ml ajoene and with the three lower ajoene concentrations there were only a few genes for which expression was significantly altered. Three of the genes are encoding components of a type VI secretion system (*tagQ1*, PA0070; *tssB1*, PA0083; *hcp1*, PA0085), and the other two genes are (*exaC*, PA1984) and (PA0182) which is a probable short-chain dehydrogenase. Among the genes significantly down-regulated by ajoene were the following QS regulated important virulence factors, LasA protease (*lasA*, PA1871), chitinase (*chiC*, PA2300), the cytotoxic galactophilic lectin (lecA, PA2570), the rhamnosyl transferase AB operon (*rhlA*, PA3478 and *rhlB*, PA3479), the PvdS-regulated endoprotease (*prpL*, PA4175) that degrades casein, elastin, lactoferrin, transferrin, and decorin (75), and the associated chitin-binding protein *cbpD* (PA0852) which mediates attachment to chitin-containing substrates and presumably assist in biofilm formation (26) (Table 1). None of the treatments seemed to affect transcription of the genes encoding central regulatory genes of the QS circuit. This is similar to other previously published QSI compounds including furanone C-30 (33), patulin and penicillic acid (59), and indicates that interaction of the inhibitor with its target may occur at the post-transcriptional level.

To verify the microarray data, RT-PCR was performed with the two stringently QS-regulated genes *lasB* and *rhlA* (Fig. 3). When comparing the two experimental methods, repression of the two genes followed the same trend, with a slightly stronger reduction observed with the RT-PCR based method. The RT-PCR data showed that a concentration of 80 µg/ml ajoene lowered expression of *rhlA* almost 12-fold and *lasB* almost 5-fold. According to Rasmussen et al. (59), the genes listed in table 1 are (except for *prpL* which is exclusively regulated by the Las QS system) subject to regulation by both the Las and Rhl QS systems. This suggests that ajoene may primarily target the Rhl system.

Attenuation of rhamnolipid production by ajoene

To exemplify the actual efficacy of ajoene in down regulating one of the important virulence factors, the concentration of rhamnolipid present in the cultures grown for DNA array and RT-PCR were directly quantified by LC-MS. The production of rhamnolipids encoded by the *rhlA, rhlB* and *rhlC* genes (PA3479, PA3478 and PA1131) is initiated in early stationary phase and coordinately regulated by the Rhl and the PQS systems (57, 73). Samples where therefore retrieved at an OD_{600nm} of 1.5 and 2.0 to monitor rhamnolipid production before and after the synthesis was fully induced. The data showed clearly that there was an increase in rhamnolipid production from an OD_{600nm} from 1.5 to 2.0. The concentrations of rhamnolipid in the samples correlated inversely with increasing concentrations of ajoene. When treated with 20 µg/ml ajoene at an OD_{600nm} of 2.0, the rhamnolipid content were reduced to approximately 1/3 compared to

459 the untreated culture and there was almost no detectable rhamnolipid present in the
460 sample when the cells were treated with 80 µg/ml ajoene (Fig. 4).
461
462 ## Effect of ajoene on *P. aeruginosa* QS signal molecule production
463 According to the transcriptomic analysis the genes inhibited by ajoene treatment could
464 indicate a posttranscriptional effect on gene products responsible for generation of C4-
465 HSL. To test this, the concentrations of C4-HSL and 3-oxo-C12-HSL in untreated and
466 ajoene-treated cultures were determined by HPLC-MS/MS at three different cell densities
467 (OD_{600nm} at 1.5, 1.8 and 2.0). The concentration of C4-HSL was found to inversely
468 correlate with increasing concentration of ajoene and at a concentration of 80 µg/ml of
469 ajoene the level was reduced almost 3-fold compared to the untreated control. With
470 respect to the concentration of 3-oxo-C12-HSL, there was no consistent effect with
471 increasing concentrations of ajoene (Fig. 5).
472
473 ## Ajoene treatment renders *in vitro* biofilms rhamnolipid deficient and thereby
474 ## prevents the killing of PMNs
475 The effect of ajoene on rhamnolipid production prompted us to investigate whether this
476 would in fact inhibit lysis of PMNs. *P. aeruginosa* biofilms were grown for four days
477 either in the presence or absence of 100 µg/ml ajoene. When freshly isolated PMNs were
478 subsequently introduced into the flow chambers of the biofilms grown in the absence of
479 ajoene, propidium iodide staining (PI) indicated extensive necrosis of the PMNs (Fig.
480 6A). In contrast, when the biofilm were grown in the presence of ajoene prior to PMN
481 exposure, no necrosis of the PMNs was observed (Fig. 6B).

Ajoene enhanced tobramycin effect on *P. aeruginosa* biofilm

We have previously published an *in vitro* treatment study of biofilms grown in the presence of QSI bioactivities including those associated with garlic extract and furanone C-30 where QS inhibition was found to greatly enhance the antimicrobial effect of tobramycin (8, 33). Biofilms of *P. aeruginosa* strain were grown either in the presence or absence of 100 µg/ml ajoene. At day three the biofilms were treated with 10 µg/ml tobramycin for 24 h. A pilot study on biofilms grown in the absence of ajoene indicated that treatment with 10 µg/ml, 100 µg/ml nor 340 µg/ml tobramycin showed no difference in the extent of killing as judged from live dead staining and inspection by means of confocal scanning laser microscopy. Our analysis showed a more than 90% killing of cells when the biofilms were grown in the presence of ajoene and subsequently treated with 10 µg/ml tobramycin (Fig. 7A). The synergistic effect was also evaluated on the clinical CF isolate CF438 (a first isolate from a diagnosed CF child), which possesses functional QS systems and once again extensive killing of the biofilm was recorded (Fig. 7B).

Antimicrobial effects *in vivo*

We performed three individual *in vivo* treatment experiments with ajoene in a pulmonary infection model in mice using 25 µg ajoene g^{-1} BW. When combining the experiments a significant difference on day three was seen ($p<0.002$) with a more than 500-fold difference recorded in clearance between the groups (Fig. 8). Experiments with a *lasR*

504 *rhlR* double mutant showed that this is the maximum obtainable difference in clearance
505 that can be obtained in this infection model (6).
506
507 Treatment of mice infected with clinical CF isolates
508 In CF patients, the appearance of the mucoid phenotype is considered a clinical marker
509 for the onset of the chronic infection, which correlates with a poor prognosis for the
510 infected CF patients. To test the efficacy of ajoene treatment on a QS proficient mucoid
511 *P. aeruginosa* strain, we adopted a clinical isolate from a patient who had a chronic
512 infection with *P. aeruginosa* for 16 years (44, 72). After administration of 25 µg/g ajoene
513 we noted an induction of abscesses, which resulted in an increase in the bacterial load in
514 the lungs. This phenomenon was not observed with the placebo group. The experiment
515 was performed twice with similar results. We then repeated the experiment with 12.5
516 µg/g and 6.25 µg/g ajoene treatments. The concentration of 12.5 µg/g gave the best
517 results with respect to bacterial clearance and apparent wellbeing of the mice, but the
518 clearance in the treatment group was not significantly different from the placebo group
519 ($p<0.5$). Treatment with 6.25 µg/g of ajoene did not have any significant effect on
520 clearance when compared with the placebo group (data not shown). An isogenic,
521 nonmucoid derivative of the clinical isolate was also investigated in a treatment study
522 using 12.5 µg/g of ajoene. This isolate cleared very rapidly from the lungs of the mice.
523 Therefore the experiment was evaluated at day one after infection. The difference
524 between the treated group and the placebo group showed a non-significant ($p<0.1$) two
525 orders of magnitude reduction (data not shown). Both isolates have a functional QS
526 system (44). To investigate the efficacy of ajoene on an early clinical *P. aeruginosa*

527 isolate, an experiment with the first isolate of *P. aeruginosa* from a CF patient was
528 conducted by treating the infected mice with 12.5 µg/g of ajoene. The experiment was
529 evaluated at day one since the strain is highly virulent and therefore the mice would not
530 survive for a three-day period. There was a significant difference ($p<0.05$) between the
531 treated group and the placebo group and a 20-fold difference (data not shown). The
532 isolate were tested positive for functional QS system (data not shown).
533

534 ## Stability of ajoene under experimental conditions

535 To test the stability in the presence of bacteria and whether ajoene could interact directly
536 with the QS signal molecules, 50 µg/ml of ajoene was incubated over night at 37°C and
537 4°C with *P. aeruginosa* (PAO1), QS-deficient Δ*lasI rhlI*, Δ*lasR rhlR* mutant, pure C4-
538 HSL, pure 3-oxo-C12-HSL and a sample with no additions. Following the incubations,
539 ajoene activity in the supernatant was assessed by means of a *lasB-gfp* dose-response
540 bioassay. The activity in the supernatant disappeared after incubation with bacteria at
541 37ºC overnight whereas there was no decrease in ajoene activity when incubated with
542 bacteria at 4ºC or incubated in the absence of bacteria at 4ºC and 37ºC. There was no
543 change in ajoene activity when incubated with pure C4-HSL and 3-oxo-C12-HSL, which
544 indicates that ajoene is not likely to chemically react with the signal molecules and cause
545 their inactivation (data not showed).
546

547 ## Effect of BSA on the bioactivity of ajoene

548 Because of putative annihilating effects of serum proteins on the bioactivity of ajoene,
549 concentrations ranging from 12.5 µg/ml to 50 µg/ml of ajoene were incubated with

bovine serum albumin (BSA). There was no notable reduction in the QSI activity of ajoene in the range of concentrations tested with serum albumin in concentrations up to 50 mg/ml, i.e. the concentration, which corresponds to the content in adult serum (see *Supplemental material*, Fig. S2).

Effects of ajoene on human lung epithelial cells

A549 human lung epithelial cells were employed as a model system to quantify potential apoptosis-inducing effects. According to cytometric determination of cleaved PARP as a marker, ajoene promotes apoptosis with an EC_{50} in the range of 100 µM (23 µg/ml). The PARP-activating effect of ajoene on lung epithelial cells was about tenfold weaker than that of positive control tetrandrine, a natural compound used in Chinese medicine for the treatment of lung disorders such as silicosis (12). Consistent with these findings, ajoene inhibited proliferation of A549 cells with an IC_{50} in the range of 100 µM and is about one order of magnitude less potent in impairment of proliferation than the control compound tetrandrine. General cytotoxicity in terms of induced cytolysis was determined by the release of lactate dehydrogenase (LDH), and, hence, LDH activity, from damaged A549 cells. Ajoene induced LDH release in a dose-dependent manner with an EC_{50} of 200 µM. It was found approximately tenfold less cytotoxic than the established furanone QS inhibitor compound C-30 (see *supplemental material*, Fig. S3A-D). Taken together, results from three independent assays coherently characterize ajoene as a compound with relatively weak interference with cell physiology of the lung epithelium.

Discussion

573 Intriguingly, worldwide emerging problems of infection control parallels a marked
574 slowdown in the development of new antibiotics. Many pharmaceutical companies no
575 longer have antibiotic drugs in the pipeline or research activities in the field. There are
576 both strategic and economic reasons for this, but there have been profound scientific
577 difficulties for the pharmaceutical industry in this context. One major limiting factor is
578 that the study of free-living, planktonic bacteria has provided the basis for our general
579 understanding of microbial life and in particular infectious diseases. Consequently, in the
580 traditional design of antibiotic drugs, it is not appreciated that the biofilm habitat may
581 dominate in chronic infections just as it does in the environment. Antimicrobial treatment
582 of biofilms is a challenge, in particular when it comes to heterogeneity, which is likely
583 one of the special features that provide biofilm bacteria such remarkable resilience.
584 The archetypical biofilm disease has for a long time been considered to be the *P.*
585 *aeruginosa* infection in CF patients. Furthermore, a recent investigation by us pointed out
586 that the early colonizers of CF children have functional QS systems. The first system lost
587 is the Las but the strains are still able to express rhamnolipid and other PQS and C4-HSL
588 controlled genes (7). Therefore, strategies that help in disabling the protective
589 mechanisms of *P. aeruginosa*, in particular the rhamnolipid shield and eDNA production,
590 are likely to be employed as a worthwhile addition to conventional antimicrobial
591 chemotherapy. Different approaches can be used to identify and harness the QS inhibitors
592 obtained from natural sources. Extraction of natural products has provided several
593 positive QSI molecules among them the *Delisea pulchra* furanones (32) and patulin from
594 *Penicillium coprobium* (59). It is interesting that sulfur-containing compounds appear as
595 a new class of molecules capable of inhibiting QS (3, 5). Li et al. (40), found two sulfur

596 containing compounds from an *in silico* based virtual screening to target the AI-2 QS
597 system from *Vibrio harveyi* and several analogues were identified to exert bioactivity
598 (52). These molecules contain a sulfone group in contrast to ajoene, which contains
599 disulfide and sulfinyl groups. For a more detailed description of identified QSIs please
600 see Galloway et al. (29).
601 We have shown that approximately 80 µg/mL (341,6 µM) ajoene efficiently switches off
602 the expression of the *rhlA-gfp* fusion in our dose-response bioassays and down regulates
603 the *rhlA* gene expression extensively which corresponds to the decrease in concentration
604 of rhamnolipid directly measured by HPLC. The gene *rhlA* encodes a
605 rhamnosyltransferase, which catalyses a glycosyl transfer reaction in rhamnolipid
606 synthesis. Rhamnolipids are glycolipids that have strong surfactant abilities. One
607 rhamnolipid in particular, rhamnolipid B, is one of the two more abundant rhamnolipids
608 and has been shown to cause necrosis of PMNs (36). Previously published data with a
609 *ΔrhlA* mutant (71) (which is only defective in rhamnolipid synthesis) make us strongly
610 believe that rhamnolipid is responsible for the lytic killing of the PMNs, also *in vivo*. We
611 have shown that ajoene treatment of *in vitro* biofilms prevents the killing of PMNs. These
612 experiments furthermore suggest that ajoene treatment is capable of attenuating the
613 production of rhamnolipids. In addition, ajoene treatment is capable of rescuing the
614 PMNs and likely to restore the action of the PMNs. The same results were observed with
615 a *ΔrhlA* mutant (71) – almost no necrotic PMNs were detected when exposed to biofilms
616 of the rhamnolipid-deficient mutant.
617 One important issue in the treatment of bacterial biofilm infections is the lowered
618 effectiveness of administered antibiotics. An infection in the airways of CF patients will

result in high concentrations of anionic polyelectrolytes like DNA (14) released from lysed inflammatory cells such as the PMNs and bacteria. It has been shown that anionic polyelectrolytes, in particular DNA, bind to and reduce the activity of cationic antibiotics like tobramycin (74, 78) which can lead to a decrease in the biological availability of tobramycin to as low as 5% of the existing dose (43). This suggests that by blocking the production of eDNA it is possible to attenuate the otherwise subsequent inactivation of tobramycin. We have shown *in vitro* that addition of 100 µg/ml ajoene to a biofilm followed by addition of 10 µg/ml tobramycin kills more than 90% of the biofilm bacteria whereas the presence of only tobramycin or ajoene had no effect. It is documented that the release of bacterial eDNA is controlled by QS (2) which, taking into consideration and combined with our results, points to a possible attenuation of the release of eDNA by ajoene. This synergistic effect is also relevant *in vivo*. It has been demonstrated that treatment of an *in vivo P. aeruginosa* foreign-body biofilm infection with a combination of a QSI and tobramycin likewise showed a synergistic clearing effect on the bacteria (16). The results could be obtained with the use of either of the QSIs: Furanone C-30, ajoene or horseradish juice extract. In addition, rhamnolipid mediated lysis of attacking PMNs may significantly contribute to the tobramycin annihilating effects *in vivo*. Our data indicate that this chain of neutralizing events may be obstructed by treatment with QSIs including ajoene. A recent study showed similar promising results with a treatment of a *Burkholderia cenocepacia* infection with a combination of tobramycin and the QSI baicalin hydrate in a mouse pulmonary model of infection (13). Several published papers by our group have demonstrated that QS-deficiency (either by mutation or by QSI treatments) leads to faster clearing compared to bacteria with functional QS in a

642 pulmonary infectious mouse model (6, 8, 33). In this study ajoene was administered
643 prophylactically and continued after infection. Enumeration by plate counts showed a
644 significant difference between the treated group and the control group on day three. This
645 is concordant with the results obtained by Bjarnsholt et al. (8), in which raw garlic extract
646 was used as treatment. In addition, a study by Harjai et al. (31), where garlic extract was
647 given to mice orally, showed significantly reduced renal bacterial content of *P.*
648 *aeruginosa* at day 5 post infection.
649 Three different clinical isolates were tested in the lung model to demonstrate the efficacy
650 of ajoene towards different isolates retrieved from CF patients. Ajoene treatment of a QS-
651 proficient mucoid strain obtained from a CF patient chronically infected for 16 years and
652 the isogenic nonmucoid strain did not have any significant effect when compared to the
653 placebo group whereas an ajoene treatment of a first CF isolate showed a significant
654 difference. This increased susceptibility of the early clinical isolate to the QSI induced by
655 ajoene is in line with our demonstration of the predominance of intact QS in early isolates
656 from CF patients (7). These data were obtained after one day post infection because of
657 difficulties in keeping the mice in a proper healthy condition. Furthermore, were the
658 ajoene concentration lowered two-fold compared to the studies with wild type. These
659 modifications (which were taken to comply with ethical constraints) in the experimental
660 procedure might be the reason for the lower effectiveness of ajoene we obtained with the
661 clinical strains compared to wild type. When comparing the present studies with ajoene to
662 earlier studies with garlic extracts, the present study offers a convincing indication of
663 ajoene being the major active component in garlic able to reduce a *P. aeruginosa*
664 infection. Two other sulfur containing molecules previously isolated by us from garlic

were found to posses QS-inhibitory activity towards the *V. fisheri* LuxR QS system, but not against the *P. aeruginosa* QS systems (53).

The question is whether it would be possible to obtain the promising treatment results in clinical trials performed on patients suffering from *P. aeruginosa* infections. The ajoene content in garlic is found in concentrations up to 172 µg/g of (E)-ajoene and 476 µg/g of (Z)-ajoene as judged from rice oil heated (80°C) freshly prepared garlic extracts (46). To match this relatively low herbal content of ajoene with the dosages required for the present animal treatments, it would require individuals to intake approximately 5 kg of raw garlic per day. Despite this, a recently published pilot study investigating the effect of garlic capsules orally administered to CF patients reported a non-significant, but never the less reduced decline in lung function (FEV_1) in the treated group compared with the corresponding placebo group (65). The exact amount of ajoene present in the capsules was not determined. However, water extracts made directly on the content of the capsules showed bioactivity directed against our *lasB-gfp* reporter (not shown). It remains therefore unknown if ajoene was present in biologically relevant amounts or whether the capsules contained ajoene-enhancing components that would increase the effects of low amounts of ajoene. In fact, we found that synthesized ajoene upon subsequent purification close to 100% purity as determined by LC-DAD-MS actually lost activity in the *in vivo* infectious models (data not shown). For example, fresh garlic extract shows a much more pronounced effect on the transcriptome of *P. aeruginosa* compared with synthesized ajoene (58). In contrast to our garlic extracts previously used, our transcriptome analysis revealed that synthetic ajoene only affected a few but nevertheless important QS controlled genes including *lasA*, *chiC* and *rhlAB,* whereas *lasB* was not

688 more than 5-fold down regulated. The effect on primarily QS-controlled genes and the
689 small amount of genes affected, suggests that synthetic ajoene, in contrast to our previous
690 garlic extracts, only inhibits expression of a minor part of the QS regulon. In comparison,
691 with more than 80% of the QS controlled genes down-regulated by furanone C-30
692 treatment and no effect on signal generation, it is unlikely that ajoene targets both LasR
693 and RhlR. Sonnleitner et al. (66), have investigated the influence of the SM-like RNA-
694 binding protein Hfq on the QS system. They documented a decrease in elastase, catalase
695 and pyocyanin production in an *hfq* knock out mutant (66) and confirmed by
696 transcriptome analysis that the effect on this subset of QS regulated virulence factors was
697 mediated by a reduced expression of the corresponding genes (67). The authors suggested
698 the following interactive path of Hfq and the QS system: Hfq binds to and stabilizes the
699 regulatory RNA RsmY, which subsequently binds to the RsmA protein, which, in turn,
700 negatively regulates RhlI messenger translation. Furthermore, they showed a decrease in
701 the concentration of C4-HSL in both a PAO1*rsmY*$^-$ and a PAO1*hfq*$^-$ strain compared to
702 the wild type (67), which corresponds to our investigations where the amount of C4-HSL
703 in cultures decrease with increasing ajoene concentrations. Furthermore, when comparing
704 our transcriptome analysis with the transcriptome analysis of the PAO1*hfq*$^-$ strain, there is
705 a compelling correlation with genes, which are significantly down regulated by ajoene
706 treatment. It is however not trivial to compare the two studies. For example, PAO1h*fq*$^-$
707 showed a reduced growth rate compared to the wild type (66). Furthermore, the authors
708 grew their samples in LB medium and isolated RNA from cultures grown to an OD_{600nm}
709 of 2.5. In comparison, our samples, which were retrieved at an OD_{600nm} of 2.0, were
710 grown in AB-minimal medium supplemented with 0.5 % Casamino acids. Despite this,

711 we suggest that either the Hfq protein or the RNA RsmY may constitute a possible target
712 of ajoene. Ajoene effects on lasB transcription as monitored by RT-PCR and DNA array
713 analysis show only a minor effect in contrast to the observed effects on on *lasB-gfp*
714 expression. We see a strong reduction of fluorescence with the *lasB-gfp* reporter with
715 increasing ajoene concentrations. The reporter strain is a translational fusion and reflects
716 the reported effect of the small regulatory RNA molecules on post-transcriptional levels
717 (as reported as reduction in elastase production by (66)). This is in support of our
718 hypothesis of ajoene targeting Hfq and the regulatory RNA's and the effect on
719 transcription of the lower parts of QS hierarchy is mediated by reduction in BHL
720 concentration. Furthermore, no effect on the transcription of *lasI* and *rhlI* is found on the
721 transcriptomic analysis, which supports the view that ajoene reducing effect on RhlI
722 expression is posttranscriptional. The much more pronounced effect of garlic extracts on
723 a multitude of QS-controlled gene expression reported by us previously (58) taken
724 together with our shortcomings in the extraction of hydrophilic compounds suggests that
725 garlic may in fact contain a multitude of QSI compounds or stabilizing agents that may
726 act in synergy and thereby in concert cover a much larger spectrum of QS controlled
727 virulence gene expression. If true, food attainable quantities of garlic in the diet may in
728 fact contribute to a natural prophylaxis against bacterial infections.
729
730 To address the question whether synthesized ajoene constitutes a pharmaceutically
731 relevant drug candidate, we investigated toxicity effects on human epithelium cells.
732 Ajoene exerts pro-apoptotic, anti-proliferative and cytotoxic effects on A549 lung
733 epithelial cells. The concentrations showing half-maximal effects in our assays were in

734 the range of 23-46 μg/ml (100-200 μM). Compared to tetrandrine, a substance used as a
735 lung therapeutic agent in Chinese medicine, ajoene is clearly less toxic (by a factor of 10)
736 towards respiratory epithelial cells. Interestingly, ajoene is less toxic for A549 cells than
737 for HL-60 leukemia cells, which respond by intense apoptosis already at concentrations
738 in the range of 4.7 μg/ml (20 μM) (23). In comparison, the concentration we used in the
739 mouse experiments was 25 μg/g (107 μM). To more thoroughly evaluate the potential of
740 ajoene as a putative component of future CF medicine, it will be important to compare its
741 cytotoxic effects with those induced by antibiotics administered in the treatment of the
742 CF syndrome.
743
744 In conclusion, we have demonstrated the use of synthetic ajoene to attenuate virulence of
745 *P. aeruginosa* by lowering expression of important QS-controlled virulence genes in *P.*
746 *aeruginosa*. It is shown for the first time that successful antimicrobial treatments with the
747 QS systems as target can be obtained by inhibiting only a few, but important virulence
748 genes and not the entire QS regulon (mediated through LasR and RhlR). This new finding
749 leads us to suggest that within the framework of QS inhibition as an antimicrobial
750 strategy small regulatory RNA molecules operating in the lower part of the QS hierarchy
751 may constitute a new, functional antimicrobial drug target. At the present, this possible
752 novel antimicrobial target needs to be extensively pursued and confirmed by molecular
753 approaches. Interestingly, small regulatory RNA's or microRNAs and their cognate
754 targets are strongly implicated in cancer, either as oncogenes or tumor and metastasis
755 suppressors. Targeting small regulatory RNAs towards therapeutic antimicrobial ends
756 would therefore parallel future developments in anticancer therapy with cancer-specific

757 miRNAs to be exploited not only to produce a direct anticancer effect but also to improve
758 the response of tumor cells to conventional treatments (35, 69). Similarly, the biofilm
759 weakening properties of ajoene with respect to enhancing the effect of conventional
760 antibiotics such as tobramycin may become instrumental for the future development of
761 combinatory treatments. It is worth to acknowledge that QS-inhibition does not remove
762 the *P. aeruginosa* capability of forming a biofilm. However, all available data indicate
763 that a QS-deficient biofilm is more fragile compared to the QS proficient biofilm (6).
764 Since for example the matrix component DNA is missing (matrix production is C4-HSL-
765 RhlR controlled) the biofilm is sensitive to shear forces and can slough off dependent on
766 the hydrodynamic forces. In addition, since rhamnolipid is not formed, the biofilm
767 becomes sensitive to the action of PMNs (because the PMNs are not killed when they get
768 in contact with the biofilm). We have previously shown that *in vitro* biofilms of QS
769 deficient bacteria can be phagocytosed by freshly isolated PMNs in contrast to QS
770 proficient biofilms (6). Central in our model for biofilm tolerance to PMNs is that
771 rhamnolipid production forms a protective shield against the incoming PMNs and we
772 have several data supporting this (1, 36, 71). Using QSIs should therefore greatly enhance
773 the antimicrobial properties of the PMNs and allow them to efficiently eradicate biofilm-
774 forming bacteria. Furthermore, rhamnolipid lyses the PMNs that subsequently spill out
775 their content of DNA, hydrolytic enzymes and oxygen radicals. This creates an “evil
776 circle” particularly with respect to tissue damage, increasing inflammation and induction
777 of mutations in *P. aeruginosa* for which the appearance of the mucoid phenotype
778 significantly contributes to exacerbations. The key thing is that this should never happen
779 and a QS-inhibitory drug should prevent this from happening. Our animal experiments

780 with clinical isolates suggest that late mucoid isolates are not sensitive to the blocking of
781 QS-controlled phenotypes whereas early isolates are likely to show sensitivity. Ajoene
782 might be able to prevent initial adherence and colonization of *P. aeruginosa* and this
783 treatment strategy might prevent the chronic lung infection by mucoid strains of *P.*
784 *aeruginosa* in CF patients. The decrease in infection in the mouse experiments, the
785 removal of *in vitro* biofilms in a combinatorial experiment with tobramycin and the initial
786 toxicity test with ajoene suggests the potential of using ajoene as a future antipathogenic
787 drug for treatment of chronic *P. aeruginosa* infections.
788

789 ## Acknowledgement

790 This work was supported by grants from the German Mukoviszidose Ve, the Danish
791 Strategic Research Council and the Novo Nordisk Foundation to MG.
792

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1062 **Figure legends:**

1063 Fig. 1: 1. Ajoene, present in two isomers; (E) and (Z). 2-5. Ajoene derivatives.

1064

Fig. 2: Expression of QS controlled specific fluorescence (Gfp expression/cell density). The QS bioassays used were *P. aeruginosa* harboring either the *rhlA-gfp* or the *lasB-gfp* fusion and *E.coli* harboring the *luxI-gfp* fusion incubated with synthesized ajoene.

Fig. 3: Fold change in gene expression of *rhlA* and *lasB* measured by RT-PCR (dark grey bars) and DNA microarray (light grey bars). Data represent the average of tree individual experiments. * indicates $P<0.05$, Student's t-test. Error bars are mean ± SD.

Fig. 4: Total rhamnolipid concentration in untreated (no add) and ajoene treated planktonic grown *P. aeruginosa.* The cultures were grown in medium supplemented with 10 µg/ml, 20 µg/ml, 40 µg/ml and 80 µg/ml of ajoene (rhamnolipid is below the detection level for the 40 µg/ml and 80 µg/ml ajoene treatment at OD=1.5). Samples retrieved at OD_{600}=1.5 (dark gray bars), and at OD_{600}=2.0 (light grey bars). Data represent the average of tree individual experiments. Error bars are mean ± SD.

Fig. 5: Concentrations of C4-HSL and 3-oxo-C12-HSL in untreated (no add) and ajoene treated planktonic grown cultures of *P. aeruginosa.* The cultures were grown in medium supplemented with 10 µg/ml, 20 µg/ml, 40 µg/ml and 80 µg/ml of ajoene. Samples retrieved at OD_{600}=1.5 (dark gray bars), at OD_{600}=1.8 (light grey bars), and at OD_{600}=2.0 (medium grey bars).

Fig. 6: Combined fluorescence and light microscopic investigations of biofilms of *P. aeruginosa* exposed to PMNs (arrow) at day four for 180 min at 37°C and then

subsequently stained with the DNA stain PI. A) The biofilm has grown without ajoene in the medium. B) The biofilm has grown in the presence of 100 µg/mL ajoene in the medium. Red fluorescence indicates lysed PMNs and green fluorescence indicates *P. aeruginosa* biofilm.

Fig. 7: Biofilms of *P. aeruginosa* PAO1 (green) (A) and a clinical *P. aeruginosa* isolate CF438 (green, stained with syto9) (B) at day four after 24 h with 10 µg/ml tobramycin or 100 µg/ml ajoene treatment for 4 days and a combination of tobramycin/ajoene. Dead cells are stained with the DNA stain PI (red). The yellow colour reflects a mixture of live and dead cells. The biofilm were visualized with confocal scanning laser microscopy.

Fig. 8: Combined results of three separate experiments of ajoene treatment versus no treatment (placebo) using the pulmonary infectious mouse model. The BALB/c mice were intracheally challenged (at day 0) with alginate beads containing $1.5x10^8$ CFU/ml *P. aeruginosa.* The two groups of mice were either untreated (placebo) or treated with ajoene 25µg/g BW once a day. The mice were given two days of prophylactic treatment or placebo. Mice were sacrificed on day one or day three post-infection and the contents of bacteria in the lungs were determined. The median values are indicated with a filled black square. The statistic significance of difference in clearance was tested by a Mann-Whitney U test (analysis of nonparametric data) and p-values for the difference at day one and day three were 0.9 and 0.002 respectively.

1110 Table 1: Alterations in gene expression by ajoene. Genes included are >5 times down
1111 regulated by 80 µg/ml ajoene treatment. The numbers are fold change in gene expression
1112 compared to an untreated control. Data represent the average of tree individual
1113 experiments. * Indicates $P<0.05$, ** indicates $P<0.01$, Student's t-test.

			Ajoene µg/ml					
Gene number	**Gene**	**Description**	**10**	**20**	**40**		**80**	
PA0852	*cbpD*	Chitin-binding protein	-2.8	-2.5	-3.9	*	-6.9	*
PA1871	*lasA*	LasA protease precurser	-2.6	-2.2	-3	**	-8.7	**
PA2069		probable carbamoyl transferase	-2.3	-2.4	-4		-5.3	*
PA2146		Conserved hypothetical protein	-1.3	-1.8	* -2.6	*	-7.3	**
PA2300	*chiC*	Chitinase	-2.5	-2.1	* -5.1	**	-24.6	**
PA2570	*pa1L*	LecA	-1.8	-2	-3.3		-6.3	*
PA3478	*rhlB*	Rhamnosyltransferase chain B	-2.6	-2	-3.3	**	-8.7	**
PA3479	*rhlA*	Rhamnosyltransferase chain A	-2.2	-1.5	-2.6		-8.8	**
PA4141		Hypothetical protein	-1	1.1	-1.3		-5.4	**
PA4142		probable secretion protein	-2	-2.2	-2.7		-5.1	*
PA4175	*prpL*	Pvds-regulated endoprotease	-3.7	-3.3	-5.3	*	-6.8	*

1114